

Title	Kinetoplastid flagellates overlooked by universal primers dominate in the oxygenated hypolimnion of Lake Biwa, Japan.
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Citation	FEMS microbiology ecology (2015), 91(8)
Issue Date	2015-08
URL	http://hdl.handle.net/2433/207708
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Type	Journal Article
Textversion	author

Title Page

Kinetoplastid flagellates overlooked by universal primers dominate in the oxygenated hypolimnion of Lake Biwa, Japan

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Keywords: 18S rRNA gene/ CARD-FISH/ clone library/ kinetoplastids/ lakes/ nanoflagellates

Running Title: Dominance of kinetoplastid flagellates in a Lake

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Abstract

Kinetoplastid flagellates, microscopically often detected from various aquatic environments and considered ubiquitous are seldom reported in molecular diversity studies with universal eukaryote DNA primers. To investigate this inconsistency, we examined nanoflagellate diversity in Lake Biwa, Japan by 18S rRNA gene clone libraries using universal eukaryote and kinetoplastid-specific primers. We also examined the abundance of kinetoplastids by catalyzed reporter deposition–fluorescence *in situ* hybridization. No kinetoplastid sequences were detected in the universal eukaryote primers library from epilimnion and hypolimnion in different seasons. However, kinetoplastid flagellates were detected with kinetoplastid-specific probe from all of the seasons and contributed up to 11.9% and 36.0% of total eukaryotes in the epilimnion and hypolimnion, respectively. Thus kinetoplastids probably are a significant, sometimes dominant, group in the hypolimnion, contributing up to 43.7% of the total flagellates. Using group-specific primers, kinetoplastid sequences were also obtained from both epilimnion and hypolimnion library. Therefore, we attributed the inconsistency to the divergent nature of 18S rRNA gene of kinetoplastids, which lead to their un-detection in the universal eukaryote primer libraries. The present study revealed that kinetoplastids have significant ecological importance in the hypolimnion of Lake Biwa, suggesting that kinetoplastids have been overlooked in other studies using universal eukaryote primers.

Introduction

The ecological importance of nanoflagellates in the planktonic food web is well known (Azam *et al.* 1983; Caron and Goldman 1990; Sherr and Sherr 1994; Nakano *et al.* 1998, 2001), and studies using molecular techniques have revealed an unexpectedly high diversity of these organisms in various aquatic environments (Lovejoy *et al.* 2006; Massana *et al.* 2006; Stoeck *et al.* 2010; Edgcomb *et al.* 2011a). However, the population ecology (Sherr and Sherr 1992), diversity and dynamics of individual nanoflagellate groups have received relatively less attention (Logares *et al.* 2012). The scarcity of information about individual groups prohibits our understanding of the ecological role of different flagellate groups in the microbial food web (Lim *et al.* 1999; Beardsley *et al.* 2005; Piwosz *et al.* 2013).

Kinetoplastids are a ubiquitous group of protists (Simpson *et al.* 2006) with high diversity (von der Heyden and Cavalier-Smith 2005; Edgcomb *et al.* 2011b) that have a characteristic structure named kinetoplast, which is a highly modified mitochondrion with densely packed DNA (Vickerman 1976). Kinetoplastids diverged from other eukaryotes due to the massive evolutionary change of their 18S ribosomal (r) RNA gene and thus occupies a basal position in the eukaryotic 18S rRNA gene phylogenetic trees (Simpson *et al.* 2006). Studies using conventional microscopic observations have detected kinetoplastid flagellates from various aquatic environments, though not as a dominant member of the nanoflagellate community (Brandt and Sleigh 2000; Weitere and Arndt 2003; Lukes *et al.* 2014). However, in contrast to morphological studies, kinetoplastids are rarely reported in eukaryote diversity studies that use molecular techniques with the universal eukaryote 18S rRNA gene primers (Lopez-Garcia *et al.* 2002; von der Heyden and Cavalier-Smith 2005; Scheckenbach *et al.* 2010; Risse-Buhl *et al.* 2013). These techniques target a large group of organisms based on their conserved region of 18S rRNA gene.

47 These contrasting observations could be attributed to the various biases associated
48 with molecular techniques which underestimate the true diversity of microorganisms (Valster
49 *et al.* 2009; Lepère *et al.* 2010). Biases in molecular studies might arise due to number of
50 reasons, some of which are, artifacts arising during DNA extraction and plasmid ligation,
51 selectivity of gene amplification of specific groups and high copy number of 18S rRNA gene
52 of some organisms (Caron *et al.* 2009; Stoeck *et al.* 2010). The Catalyzed Reporter
53 Deposition–Fluorescence *in Situ* Hybridization (CARD-FISH) is an efficient tool to
54 circumvent the biases due to culture and molecular study and is widely used to enumerate
55 microorganisms from natural habitat (Pernthaler *et al.* 2002; Acinas *et al.* 2005). However,
56 limited numbers of probes are available for eukaryotic microorganisms relative to that of
57 bacteria (Lim *et al.* 1999; Loy *et al.* 2007), with only a few group-specific probes for
58 flagellates (Massana *et al.* 2006; Lepère *et al.* 2010; Piwosz and Pernthaler 2010). A
59 kinetoplastid group-specific probe has been constructed by correcting a mismatch in the
60 universal eukaryote probe (EUK516) sequence due to which kinetoplastids were not targeted
61 and thus underestimated in total eukaryote counts (Bochdansky and Huang 2010).
62 Information about the abundance and diversity of kinetoplastids is limited, with only a few
63 studies concerning the abundance of kinetoplastids using FISH probes in oceans (Edgcomb *et*
64 *al.* 2011b; Morgan-Smith *et al.* 2011, 2013). A surprisingly high abundance of kinetoplastids
65 was reported in all of these studies, with their increased abundance in deeper waters. In
66 addition, kinetoplastid sequences were detected from various environments including some
67 extreme environments e.g. hypersaline anoxic basins, abyssal sea floor, etc. using
68 kinetoplastid-specific primers (Scheckenbach *et al.* 2010; Edgcomb *et al.* 2011b; Salani *et al.*
69 2012).

70 The abundance and diversity of kinetoplastids in freshwater lakes are poorly known
71 due to fewer quantitative studies regarding nanoflagellates in freshwater lakes (del Campo

72 and Massana 2011). Moreover, studies have mainly focused on surface waters (Lefranc *et al.*
73 2005; Richards *et al.* 2005; Mangot *et al.* 2009; Nolte *et al.* 2010), and information about
74 deeper waters is scarce (Salbrechter and Arndt 1994; Lepère *et al.* 2010). Only one study
75 reported a high abundance of kinetoplastid-like flagellates in the hypolimnion of deep lakes
76 (Steinberg *et al.* 1983). However, the study by Steinberg *et al.* (1983) was conducted using
77 inverted microscope, and therefore no sequence data is available to compare or confirm the
78 organism observed. Information about the diversity and abundance of nanoflagellates in
79 different layers of deep lakes is important to understand the contribution and roles that
80 different flagellates play in the freshwater food web.

81 To investigate the dynamics of kinetoplastids in freshwater lakes, the present study
82 was conducted in Lake Biwa as it is deep (maximum depth 104 m) and largest freshwater
83 lake in Japan. We constructed 18S rRNA gene clone libraries using universal eukaryotic
84 primers from different depths and seasons to examine whether the method underestimates the
85 relative abundance of kinetoplastid flagellates. CARD-FISH analysis was conducted using
86 kinetoplastid-specific probes to study their seasonal and vertical abundance. Additionally,
87 clone libraries were constructed from epilimnion and hypolimnion sample using
88 kinetoplastid-specific DNA primers. The molecular analysis (clone library) and microscopic
89 analysis (CARD-FISH) were combined to understand the potential bias that is responsible for
90 the contradictory results given by the two methods to detect kinetoplastids.

91

92 Materials and Methods

93 *Study site and Sampling*

94 The study was conducted in the monomictic and mesotrophic Lake Biwa, the largest
95 freshwater lake in Japan (surface area 674 km², maximum depth 104 m). The lake has an
96 oxygenated hypolimnion with minimum dissolved oxygen concentrations generally above
97 100 µM (Kim *et al.* 2006). During winter (January-March), the water column of the lake is
98 vertically mixed due to cooling and a strong seasonal wind, whereas during the rest of the
99 year, the water column is thermally stratified (Thottathil *et al.* 2013).

100 The samples were collected from January 2012 to January 2013 on a monthly basis
101 and once on August 2013 from station Ie-1 (35° 12' 58" N, 135° 59' 55" E, depth 73 m), a
102 long term limnological survey station of Center for Ecological Research, Kyoto University,
103 Japan. The hydrographic structure was determined with a conductivity-temperature depth
104 profiler (SBE-911 plus; Sea Bird Electronics, Sea-logger, WA, USA) equipped with an
105 oxygen sensor (13 E, SBE). Water samples from six depths (5, 10, 15, 20, 50 and 70 m)
106 representing the epilimnion, metalimnion and hypolimnion were collected with a 5 litre
107 Niskin sampler (General Oceanics, Miami, USA). The samples were drawn into clean plastic
108 bottles that were rinsed three times with sample water before collection; they were kept cool
109 and dark in an icebox and transported to the laboratory within three hours of collection.

110 The chlorophyll *a* concentration was determined by filtering 150 ml of the water
111 sample through a GF/F filter (diameter 25 mm, Whatman) and analysing the sample
112 following the *N,N*-dimethylformamide fluorometric method (Moran & Porath 1980).

113 *Total count of bacteria and nanoflagellates*

114 Samples were fixed with 1% final concentration of glutaraldehyde immediately after
115 collection and were stored at 4°C until filtration. A 1 to 2 ml water sample was filtered
116 through a polycarbonate membrane filter (pore size 0.2 µm, diameter 25 mm, Advantec), and
117 stained with 4, 6-diamidino-2-phenylindole (DAPI) to enumerate the total bacteria (Porter

and Feig 1980). The bacterial cells were visualized under UV light with an epi-fluorescent microscope (Olympus BX- 50, Japan). Duplicate samples from each depth were counted at 1000× magnification from 20 randomly chosen fields (on an average, a minimum of 300 cells were counted). For the enumeration of the total heterotrophic and autotrophic nanoflagellates (HNF and ANF), 30 ml of epilimnion and metalimnion (5-20 m) and 50 ml of hypolimnion (50 and 70 m) waters were filtered through polycarbonate membrane filters (pore size 0.8 µm, diameter 25 mm, Advantec) and stained with primulin (Caron 1983). HNF and ANF cells were observed with an epi-fluorescent microscope under UV and green excitation, respectively. For each sample, 100 fields were counted at 1000× magnification (on average, a minimum of 100 cells were counted for HNF and simultaneously ANF cells were also counted in each field).

DNA extraction and Clone library analysis

The samples for the clone libraries were collected in each season from the epilimnion (5 m or 10 m) to obtain the maximum abundance of nanoflagellates (Nagata 1988) and once from the hypolimnion in August, 2013 (Table 1). Water samples were collected after pre-filtering with a 20 µm plankton net, and 1-2 litre portions of the filtrate were filtered through polycarbonate membrane filters (pore size 0.8 µm, diameter 47 mm, Costar) at low vacuum (5 cmHg) and stored at -80°C. The DNA extraction was conducted using the Power Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). The eukaryotic 18S rRNA genes were polymerase chain reaction (PCR) amplified by universal eukaryote primers EukA and EukB (Medlin *et al.* 1988). PCRs were performed in 20 µl of reaction volume with Blend Taq PCR kit (Toyobo, Osaka, Japan). The amplification was performed under the following conditions: initial denaturation at 95°C for 2 min; 35 cycles (95°C for 30 s, 59.5 °C for 30 s, 72°C for 2 min); and final extension at 72°C for 7 min. Two separate clone libraries were constructed from epilimnion and hypolimnion using kinetoplastid-specific primers kineto14F and

kineto2026R following the PCR condition mentioned by von der Heyden and Cavalier-Smith (2005) to isolate kinetoplastid sequences (Table 1). The PCR products were purified with the Exo-I and TSAP enzymes and cloned using the pT7 Blue Perfectly Blunt Cloning Kit (Novagen, Madison, WI, USA) according to the manufacturer's instructions.

Sequencing of clones and phylogenetic analysis

The sequencing of clones was performed using the Euk 528F (Elwood *et al.* 1985) primer for the V4 region (Lovejoy *et al.* 2006) and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) to obtain an 800-900 bp segment of the gene. The sequencing was performed with an ABI Genetic Analyzer 3130xl Avant Capillary automated sequencer. The quality of the sequences was assessed using the Sequencing Analysis Software v5.3.1 (Applied Biosystem) and the sequences were manually corrected and trimmed using the MEGA6 software (Tamura *et al.* 2013). The closest match to each sequence was obtained from BLAST search (Altschul *et al.* 1997), and sequences whose closest match was under 97% were checked for chimeras with additional BLASTs of several sections of the sequence. Sequences were also checked for chimeras using KeyDNATools (<http://KeyDNAtools.com>). Chimeras, low-quality sequences and sequences less than 500 bp were excluded from further analysis. Sequences affiliated with eukaryotes other than flagellates (e.g. Ciliophora, Fungi and Diatoms) were not included. The sequences were aligned using the CLUSTAL W package (Thompson *et al.* 1994). The operational taxonomic units (OTUs) were separated at 97% similarity, and the similarity matrix was calculated using Bioedit (Hall 1999). The selected clones which represented one member of each flagellate OTU were sequenced using the internal forward and reverse primers, 1055F (Holman *et al.* 2003) and D978 (Zimmermann *et al.* 2011) and EukB primer.

Kinetoplastid sequences obtained using kinetoplastid-specific primers from the epilimnion and hypolimnion were closely related to *Rhynchomonas nasuta* and *Neobodo designis* in the BLAST search, respectively. The phylogenetic analysis therefore was conducted with closely related *Rhynchomonas* and *Neobodo* sequences available in the GeneBank (<http://www.ncbi.nlm.nih.gov/>) and also with other members belonging to Neobodonida (e.g. *Cruzella marina*, *Dimastigella trypaniformis*, and *Rhynchobodo* sp.) and closely related bodonids. Six 18S rRNA gene sequences were retrieved from GeneBank affiliated to slow-evolving kinetoplastids (*Ichthyobodo* and *Perkinsiella*-like sp.) and used as outgroup (Moreira *et al.* 2004). Total 60 partial and full length sequences were aligned by PRANK algorithm with Trust insertions (+F) (Löytynoja and Goldman 2008) from 100 alternative bootstrap alignments. Ambiguously aligned positions were masked from our analysis using GUIDANCE (Penn *et al.* 2010) with specific residue cutoff value of 0.93. Maximum likelihood (ML) tree was constructed using MEGA6 software based on Kimura 2-Parameter model of nucleotide substitution with a six category discrete approximation of a Γ distribution plus invariable sites (K2+ Γ + I model) (Kimura 1980). Model test confirmed that K2+ Γ + I model was the most appropriate model for the given data. Initial trees for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. ML trees were constructed from 1000 bootstrap replicates. To further confirm the results obtained from the ML tree, Neighbor joining (NJ) analysis was performed using Maximum Composite Likelihood model with a six category Γ distribution of rate variation among sites. NJ trees were constructed from 2000 bootstrap replicates. The result of only ML tree was discussed as the topology of consensus NJ tree was the same as ML tree.

CARD-FISH

Water samples were pre-filtered through a 20 μm mesh plankton net and fixed in a 2% final concentration of formaldehyde (freshly prepared by filtering through 0.2 μm syringe filter) for at least 3-4 hours before filtration. The nanoflagellate cells were harvested on polycarbonate filters (pore size 0.8 μm , diameter 25 mm, Advantec); they were rinsed twice with 1X PBS and twice with MilliQ water, air dried and frozen at -20°C until further processing.

CARD- FISH was performed according to the protocol of Okazaki *et al.* (2013), based on Pernthaler *et al.* (2004). Permeabilization with lysozyme or proteinase K was not performed (Bochdansky and Huang 2010). The filters were embedded in 0.1% low-gelling-point agarose and cut into eight sections, which were hybridized at 35°C for 12 hours with $0.5\text{ }\mu\text{g ml}^{-1}$ concentration of probe and 30% concentration of formamide. A formamide stringency test was performed with a gradient of formamide (0-55%) with 35°C and 45°C temperatures, where optimal stringency was achieved at 35°C with 30% formamide. Optimal stringency test was conducted using a cultured kinetoplastid sp. isolated from Lake Biwa and also with hypolimnion samples from Lake Biwa. The probes, (Table 2) were purchased from the Thermo Electron Co. (Ulm, Germany). Counting was performed using an Olympus BX50 epifluorescence microscope under $1000\times$ magnification at blue/UV excitation. For the kinetoplastids, either 100 microscopic fields were counted, or when the densities were low, the complete filter piece was screened per sample. To confirm the kinetoplastid cells, the hybridized cells were counter checked under DAPI staining for the nucleus and kinetoplast (Fig. 1). The total eukaryotes were counted simultaneously with the kinetoplastid cells by DAPI staining under UV excitation.

Statistics

Spearman's rank correlation coefficient was calculated for testing the relationship between abundance of kinetoplastids with environmental and biological parameters. Bonferroni's correction was also conducted to correct the familywise error rate. The statistics were computed in the R environment (www.r-project.org).

Results

Physico-chemical and biological characteristics

The water column was completely mixed from January to March with a mean temperature of $7.6 \pm 0.5^{\circ}\text{C}$ (Fig. 2a). Stratification commenced in April, and the epilimnion water (5 m) temperature varied widely from 11.6°C in April to a maximum of 29.1°C in August and decreasing to 10.2°C in December. However, the hypolimnion water temperature remained stable with a mean temperature of $7.6 \pm 0.2^{\circ}\text{C}$ throughout the study period (Fig. 2a).

The maximum chlorophyll *a* concentration ($31.1 \mu\text{g l}^{-1}$) was detected in May (5 m) with an average concentration of $4.5 \pm 5.7 \mu\text{g l}^{-1}$ during the study period (Fig. 2b). High values remained until July, decreased drastically in August and September and increased slightly in October and November. However, the chlorophyll *a* concentration reduced from December and remained low and uniform throughout the water column during winter. The concentration of chlorophyll *a* always remained low in the hypolimnion, with the lowest concentration ($0.3 \mu\text{g l}^{-1}$) in October (50 m). The bacterial abundance was high in the epilimnion, with two peaks (maximum at 5 m in May and July with $4.6 \times 10^6 \text{ cells ml}^{-1}$ and $4.8 \times 10^6 \text{ cells ml}^{-1}$, respectively) with an average concentration of $2.5 \pm 0.9 \times 10^6 \text{ cells ml}^{-1}$ during the study period (Fig. 2c). The abundance of bacteria was relatively low in the

hypolimnion, and the lowest densities were detected from 50 m. Low and uniform bacterial abundance was found throughout the water column during the winter mixing period.

HNF and ANF abundance

HNF were abundant in the epilimnion and metalimnion during the stratified period, and a high abundance of HNF fluctuated vertically, depending on the seasons. The maximum abundance was found at 5 m in May with 36.9×10^3 cells ml^{-1} with an average concentration of $7.1 \pm 5.2 \times 10^3$ cells ml^{-1} throughout the study period (Fig. 2d). The high abundance of HNF fluctuated at 5 m in May and June, which shifted to 10 m and 15 m during July to September and shifted back to 5 m in October and November. Similarly, the peak of ANF abundance was found fluctuating between 5 m to 15 m during May to November. Maximum abundance of ANF was found at 5 m in September and 20 m in December, with 2.8×10^3 cells ml^{-1} and an average concentration of $1.0 \pm 0.8 \times 10^3$ cells ml^{-1} (Fig. 2e). Similar to the bacteria, the abundance of both ANF and HNF were low in the hypolimnion. During the winter, the HNF density was relatively low, whereas high densities of ANF were found throughout the water column.

Clone library

The five clone libraries from the epilimnion representing four seasons, and the hypolimnion yielded 403 clones (Table 1) where flagellate groups contributed to 84 unique OTUs. Cryptophyta had the highest diversity in the epilimnion nanoflagellate communities, with their highest contribution in May which accounted for 57% of the total OTUs (Fig. 3). Other groups obtained from the epilimnion libraries with significant contribution were Perkinsozoa and Cercozoa. Dinophyta were the most diverse group in the hypolimnion library and contributed to 34% of the total OTUs followed by Cercozoa, Bicosoecida and Chrysophyta.

The OTUs of various phylogenetic groups of nanoflagellates were detected including singletons. These singletons most likely represented rare taxa, whereas no sequence affiliated with kinetoplastid flagellates was obtained from the libraries. However, both epilimnion and hypolimnion libraries with kinetoplastid-specific primers yielded kinetoplastid sequences. The epilimnion library with kinetoplastid specific primers yielded four kinetoplastid sequences (Table 1) which belonged to one OTU and having the closest match with *Rhynchomonas nasuta* in the BLAST search. Whereas, hypolimnion library with kinetoplastid-specific primers yielded six kinetoplastid sequences (Table 1) which belonged to two OTUs and had the closest match with *Neobodo designis*. The phylogenetic analysis revealed that kinetoplastid from the epilimnion of Lake Biwa is related to *R. nasuta* (Clade 2, Fig. 4), having closest similarity with a *R. nasuta* strain from deep sea sediment with a low bootstrap support. The phylogenetic analysis of kinetoplastids from the hypolimnion of Lake Biwa revealed that both the OTUs were closely related to each other with 79% bootstrap support and were included in a clade (Clade 1) with *N. designis* sequences only from freshwater environment (Fig. 4). Nevertheless, kinetoplastids from the hypolimnion of Lake Biwa were separated from other closely related sequences in the clade with a high bootstrap support.

Dynamics of kinetoplastids

The CARD-FISH positive kinetoplastid cells were detected throughout the year (Fig. 5a), although, at some depths they were not detected during winter and spring. Kinetoplastid abundance was low in the epilimnion in April and May, despite the high chlorophyll *a* concentrations and high abundance of bacteria and HNF (Fig. 2). Statistical tests indicated that kinetoplastid abundance was significantly correlated ($p < 0.001$) only with chlorophyll *a* concentration (Table 3). The abundance of kinetoplastids increased in the epilimnion during the summer, with relatively high values from June to December (Fig. 5a). Two peaks of high

abundance were found, one during July and August and the other during November and December. The highest abundance of kinetoplastids in the epilimnion was recorded in December (10 m) with 9.3×10^2 cells ml^{-1} , which accounted for 11.9% of the total eukaryotes (Fig. 5b) and 12.1% of the total flagellates (HNF+ANF) (Fig. 5c). The kinetoplastid cell density was low during winter mixing throughout the water column. Interestingly, in the hypolimnion, we found that kinetoplastids were the dominant nanoplankton during summer and autumn (Fig. 5a). The abundance of kinetoplastids in the hypolimnion increased from June, with peak abundances observed in August and September. Their abundance remained high until December in both 50 m and 70 m with the maximum cell numbers at 70 m in August (1.2×10^3 cells ml^{-1}). The percentage contribution of the kinetoplastids in the hypolimnion in August (2012) accounted for 31.6% and 36.0% of the total eukaryotes (Fig. 5b) and 41.8% and 43.7% of the total flagellates (Fig. 5c) at 50 m and 70 m, respectively. The hypolimnion library in August (2013) by the universal eukaryote primers did not detect any kinetoplastid sequences, however, the percentage contribution of CARD-FISH positive kinetoplastids was 47.8% and 53.6% of the total eukaryotes at 50 m and 70 m respectively (data not shown), which was even higher than their contribution in the previous year (2012).

Discussion

We found a high abundance of HNF in the upper layers of Lake Biwa during the study period with two peaks in early summer and autumn with the highest abundance in early summer (Fig. 2d) which corroborates with the other studies in HNF (Nagata 1988; Auer and Arndt 2001; Auer *et al.* 2004; Mangot *et al.* 2009). Abundance of HNF during early summer in the present study was higher than the HNF abundance in German mesotrophic lakes (Auer and Arndt 2001), but was in the similar range of that in French oligomesotrophic and mesotrophic

lakes (Lepère *et al.* 2010). Abundance of kinetoplastids, on the other hand increased in the epilimnion during late summer and autumn (Fig. 5), and this corroborates with the study in German lakes (Auer and Arndt 2001).

Dominance of kinetoplastid flagellates in deeper waters of Lake Biwa

Kinetoplastids are considered less important in their contribution to total HNF (Arndt *et al.* 2000) and in oligomesotrophic Lake Mondsee, kinetoplastids and other flagellates were found to reduce at greater depths (Salbrechter and Arndt 1994). By contrast, in Lake Biwa, kinetoplastids were found to be the dominant member of HNF community in the hypolimnion and were also present throughout the year in the water column (Fig. 5c). The dominance of kinetoplastids in deeper layers indicated their importance in the hypolimnion HNF community characterized by a low abundance of flagellates. Thus, kinetoplastids play a major role in the matter cycling of Lake Biwa during the summer stratification because the dominant taxa have the major contribution to the ecosystem's function (Scheckenbach *et al.* 2010).

A flagellate identified as *Phyllomitus apiculatus* (Cryptophyceae) was found to dominate in the hypolimnion of some deep lakes to avoid zooplankton grazing and their maximum densities were observed just after dense diatom populations (Steinberg *et al.* 1983). The dynamics of the kinetoplastids in Lake Biwa were similar to the dynamics of *P. apiculatus*, since they were dominant in the hypolimnion in the summer and their abundance in the epilimnion increased in August and December, immediately after the chlorophyll *a* concentration was reduced. The taxonomic affinities of organisms assigned to the genus *Phyllomitus* are complex (Lee 2002), however the *P. apiculatus* reported by Steinberg *et al.* (1983) resembles *Rhynchobodo spp.*, which belongs to the class Kinetoplastidae (Vickerman 2000; Lee 2002). Therefore, the study by Steinberg *et al.* (1983) might be the first report of

the dominance of kinetoplastid flagellates in the hypolimnion of deep lakes. The kinetoplastid sequences obtained from the hypolimnion of Lake Biwa had the closest match to *Neobodo designis* in BLAST search. According to the updated kinetoplastid phylogeny (Moreira *et al.* 2004), both *N. designis* and *Rhynchobodo* belongs to the same order Neobodonida. No sequence is available for the reported organism by Steinberg *et al.* (1983), therefore phylogenetic analysis or comparison with Lake Biwa kinetoplastid is not possible, but both belonging to same species or are a close relative cannot be ruled out.

In the phylogenetic analysis, kinetoplastids from the hypolimnion of Lake Biwa belonged to a *N. designis* clade (Clade 1) that consisted of sequences from freshwater and mainly from freshwater sediments (Fig. 4). Nevertheless, Lake Biwa kinetoplastids branched from other members of the cluster with high bootstrap support. The results indicate novelty of the Lake Biwa kinetoplastid sub-cluster due to little information known about Neobodonid diversity and low amount of research conducted on free living kinetoplastids in limnetic ecosystems. However, *Neobodo* clade is a newly created group of kinetoplastids from which was earlier considered as members of *Bodo* (Moreira *et al.* 2004). *Neobodo* clade is complicated with many subclades (von der Heyden *et al.* 2004), and clear demarcation of its members is not available. It is therefore not possible to assign a specific taxonomic affinity to the Lake Biwa kinetoplastids.

Kinetoplastids had an inverse relationship with temperature during stratification (Fig. 2a, 5a), due to the dominance in the hypolimnion with low and stable water temperature (7°C). Thus, low water temperature may be favorable for the growth of kinetoplastids. However, the dominance of kinetoplastids was not observed in the water column with low water temperature during winter mixing period, therefore we could not find any significant relationship with kinetoplastid abundance and temperature (Table 3). Hence, only temperature cannot explain the dominance of kinetoplastids in the hypolimnion. On the other

hand, Salani *et al.* (2012) have reported that free-living kinetoplastids especially *Neobodo* and *Rhynchomonas* were found to be abundant in ocean abyssal depths characterized by permanent stratification with constant and homogenous environment. *N. designis* is one of the most ubiquitous freshwater flagellates (Patterson and Lee 2000) and the majority of *N. designis* strains are not tolerant to a wide variety of environmental conditions (von der Heyden and Cavalier-Smith 2005). Thus, in the present study, the hypolimnion during the stratification period having constant and homogenous environment might be the reason for the dominance of these flagellates.

The bacterial group CL500-11 (phylum *Chloroflexi*) is the dominant bacteria in the hypolimnion of Lake Biwa (Okazaki *et al.* 2013). The dynamics of CL500-11 in the hypolimnion were similar to the dynamics of kinetoplastids which suggested that similar environmental conditions may favor the growth of kinetoplastids and CL500-11 in Lake Biwa. Large size and curved shape of CL500-11 bacteria might protect them from grazing and thus allow this group of bacteria to dominate in the hypolimnion of Lake Biwa. However, some flagellates, especially bodonids are capable of feeding on large bacteria (Gonzalez *et al.* 1990; Šimek and Chrzanowski 1992; Posch *et al.* 1999). Therefore kinetoplastids might have an advantage over other flagellates in the hypolimnion due to their ability to feed on large bacteria dominant in the deeper layers of Lake Biwa.

Another possible explanation for the dominance of kinetoplastids is their grazing on deposited bacteria. Previous studies demonstrated that *Bodo saliens* (probably *N. designis* or a close relative belonging to the order Neobodonida) grew on deposited rather than suspended bacteria in laboratory systems (Zubkov and Sleigh 2000). Kinetoplastids are known to feed on attached bacteria (Caron 1987), inhabiting on macroaggregates such as *Rhizosolenia* mats and marine snow (Caron *et al.* 1982; Artolozaga *et al.* 2000). In the present study, dominance of kinetoplastids after the decrease in the chlorophyll *a*

concentration (Fig. 2b, 5) might be due to their increased abundance in detritus. This point is further clarified by the fact that kinetoplastids had only significant negative correlation with chlorophyll *a* concentration (Table 3). In the present study, the kinetoplastids, *R. nasuta* and *N. designis* identified from the epilimnion and hypolimnion respectively are known to be poor swimmers and glide over surfaces (Boenik and Arndt 2000). Therefore increased abundance of kinetoplastids in the hypolimnion water might be due to their growth in the associated detritus sinking from the epilimnion. Thus, more studies are needed to understand the factors responsible for the dominance of kinetoplastids and whether kinetoplastid flagellates prefer the oxygenated hypolimnion of deep lakes.

Kinetoplastids: a rare or an underestimated group of flagellates?

The sequences affiliated with the kinetoplastid flagellate were not detected in clone libraries using universal eukaryote primers, whereas kinetoplastid sequences were detected using kinetoplastid-specific primers from both epilimnion and hypolimnion. The CARD-FISH analysis demonstrated that kinetoplastids were present in all of the seasons and even dominant on some occasions (Fig. 5a). Kinetoplastids were also not detected in the hypolimnion library with universal eukaryote primers (Figure 3) where the abundance of kinetoplastids was high (Figure 5a). These results supported our hypothesis that kinetoplastid flagellates were underestimated when using universal eukaryote primers.

A study conducted in the groundwater of a karstified aquifer found several kinetoplastid flagellates by microscopic observation whereas no sequences were detected using universal eukaryote primers (Risse-Buhl *et al.* 2013). Kinetoplastids are also not detected by commonly used universal eukaryote FISH probes (Bochdansky and Huang 2010) due to the divergent nature of their 18S rRNA (Simpson *et al.* 2006). Thus, divergent nature of the 18S rRNA gene might be the possible reason for underestimation of kinetoplastids in the clone libraries when using the universal eukaryotic primers.

The high abundance of kinetoplastids was reported from the Atlantic Ocean using kinetoplastid-specific FISH probes (Morgan-Smith *et al.* 2011, 2013). Similarly, a high diversity of kinetoplastid flagellates was reported from various aquatic environments using group-specific primers (von der Heyden and Cavalier-Smith 2005; Rasmussen *et al.* 2001, Edgcomb *et al.* 2011b). However, diversity studies using universal eukaryote primers rarely reported kinetoplastid sequences from marine environments (Stoeck *et al.* 2010; Logares *et al.* 2012). Thus, our results suggested that kinetoplastids might have been overlooked in diversity studies in other freshwater or marine environments using universal eukaryote primers, thereby underestimating their importance in the aquatic food web.

Conclusion

This study showed that kinetoplastids are an important member of nanoflagellate community in Lake Biwa. We found that these flagellates comprised the major component of microbial eukaryote community in the hypolimnion. Previously, only one study reported dominance of kinetoplastid-like flagellates in some Austrian lakes. A possible reason for the lack of information about the kinetoplastids is the underestimation of their abundance in molecular studies when using the universal eukaryote primers, due to the divergent nature of their 18S rRNA gene. Another reason for limited information is due to lack of studies in oxygenated hypolimnion of deep lakes and this is the first study to reveal the seasonal and vertical dynamics of a particular flagellate group in a lake using CARD-FISH. Kinetoplastids may dominate in oxygenated hypolimnion of other deep lakes in common with previously reported bacterioplankton CL500-11. Studies from other deep lakes and experiments are necessary to identify the factors responsible for their dominance and to understand their role in the ecosystem.

431

Funding

432 This work was supported by Grants-in-Aid for Scientific Research [grant number 23370010]
433 from the Japan Society for the Promotion of Science and Japan Science and Technology
434 Strategic International Research Cooperative Program project ‘Fate of dissolved organic
435 matter in lakes with special reference to loading and pollution’. I. Mukherjee was supported
436 by a scholarship from the Japanese Ministry of Education, Culture, Sports, Science, and
437 Technology.

438

Acknowledgements

439 We are grateful to Prof. Ramon Massana and Dr. Kasia Piwosz for their comments. We thank
440 Tadatoshi Koitabashi and Dr. Yukiko Goda for their help during sample collection and to Dr.
441 Masayuki Ushio, Dr. Kako Ohbayashi, Yusuke Okazaki, Dr. Hiroyuki Takasu, Shoji
442 Thotatthil and Shohei Fujinaga for their help and comments.

443 *Accession numbers:* The sequences were deposited in the DDBJ nucleotide database under
444 accession numbers AB996606 - AB996691 and LC056843.

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Conflict of Interest Statement

447 The authors declare no conflict of interest.

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449

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651

651 Table 1 Number of sequences analysed and flagellate OTUs obtained from each library

Season and year	Depth	Target group	No. of sequences	Flagellate
			analysed	obtained
Spring (May) 2012	5m	eukaryotes	72	32
Summer (August) 2012	10m	eukaryotes	151	47
Autumn (October) 2012	5m	eukaryotes	79	30
Winter (December) 2012	10m	eukaryotes	30	11
Summer (August) 2013	50m	eukaryotes	71	30
Spring (May) 2012	5m	kinetoplastids	4	1
Summer (August) 2013	50m	kinetoplastids	6	2

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654 Table 2 CARD-FISH probes used in the present study

FISH probes	Sequence (5' to 3')	Position*	Target group	References
KIN 516	ACCAGACTTGTCTCC	502-517	kinetoplastids	Bochdansky and Huang 2010
EUK 516	ACCAGACTTGCCCTCC	502-517	eukaryotes	Bochdansky and Huang 2010
Competitor				
NON 338	ACTCCTACGGGAGGC	—	—	Wallner <i>et al.</i> 1993

655 *positions refer to the 18S rRNA gene of *S. cerevisiae*.

656

656

657 Table 3 Results of Spearman's rank correlation test between abundance of kinetoplastids with
 658 environmental and biological parameters. Significant probability is 0.01 after Bonferroni's
 659 correction (n=78).

	Temp.	Chl <i>a</i>	Bacteria	HNF	ANF
ρ (<i>rho</i>)	0.15	-0.37	-0.18	-0.13	-0.06
p (<i>probability</i>)	0.198	<0.001	0.108	0.244	0.615

660

661

Titles and legend to figures

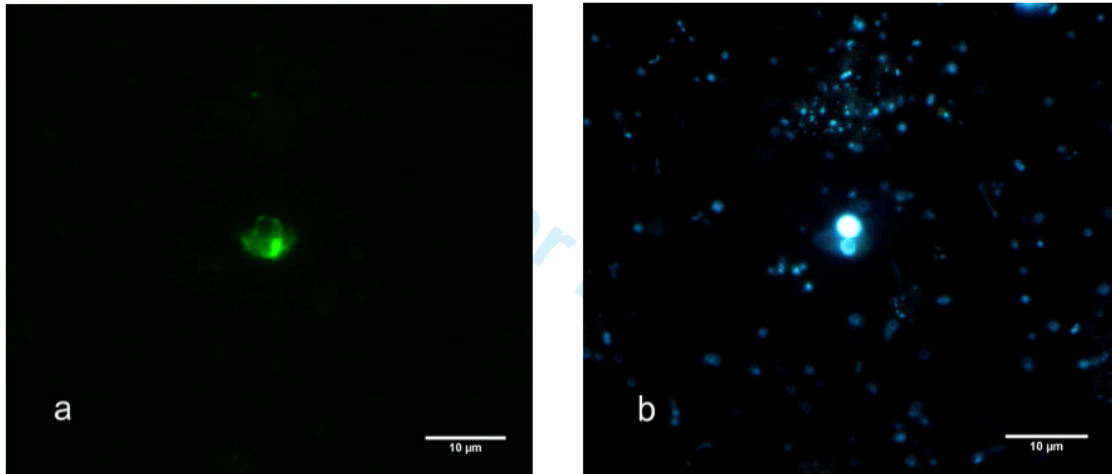


Figure 1: A kinetoplastid cell of the same microscopic field as seen under (a) blue excitation with CARD-FISH probes (KIN516 and Euk516 competitor) and (b) UV excitation showing nucleus and kinetoplast by DAPI staining. Both the images correspond to the same microscopic field taken with an attached camera at 1000× magnification. Contrast of the photos has been increased from the original microscopic image.

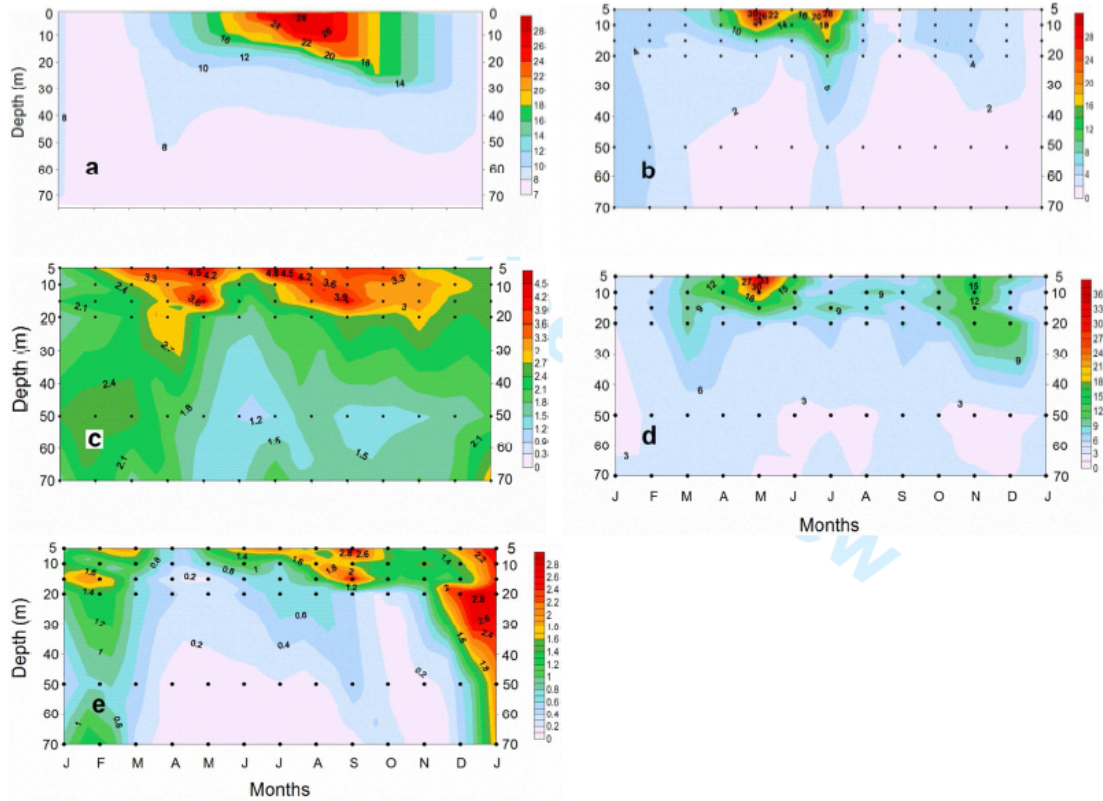


Figure 2: Seasonal changes in vertical distribution of (a) temperature (°C), (b) chlorophyll *a* (µg l⁻¹), (c) bacterial abundance (10⁶ cells ml⁻¹), (d) total HNF (heterotrophic nanoflagellate) abundance (10³ cells ml⁻¹) and (e) total ANF (autotrophic nanoflagellate) abundance (10³ cells ml⁻¹). Black dots represent sampling depth.

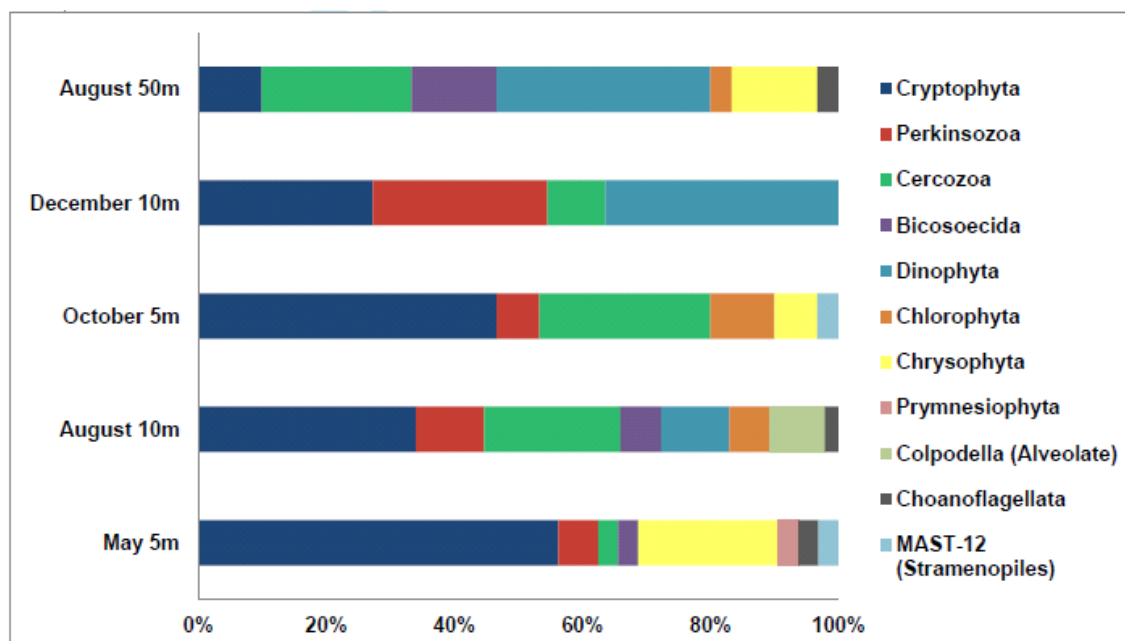


Figure 3: Seasonal changes in nanoflagellate communities by 18S rRNA gene clone library analysis based on the OTU abundance of each group. Sequences affiliated with eukaryotes other than flagellates (e.g., Ciliophora, Fungi and Diatoms) obtained in clone libraries were not included in the analysis. x axis represent group percentage of the total OTUs per sample and y axis represent the month and water depth of sample collection.

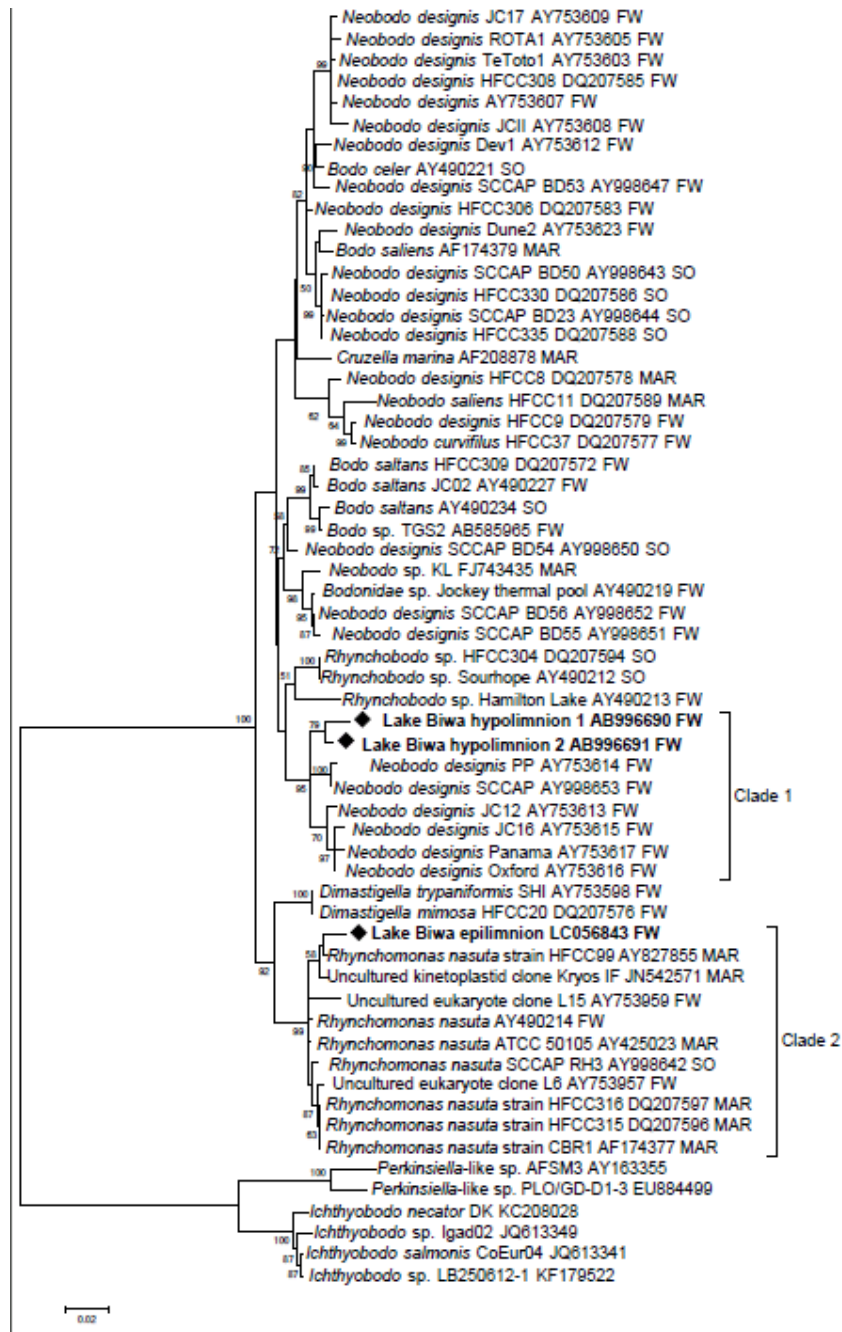


Figure 4: 18S rRNA gene phylogenetic tree of Lake Biwa kinetoplastid flagellates from the epilimnion and hypolimnion with 54 sequences of neobodonids and closely related bodonids. 6 sequences belonging to slow-evolving kinetoplastids were used as outgroup. Kinetoplastid sequences obtained from this study are highlighted in bold and marked with black diamond at the external nodes. Maximum likelihood tree was calculated using K2+ Γ + I model with 1000

686 bootstrap replicates. The bootstrap replicate support percentages > 50% is shown next to the
687 branches. Clades containing kinetoplastids from hypolimnion and epilimnion of Lake Biwa
688 are named as Clade 1 and 2 respectively. Source of each sequence is indicated by labels at the
689 end of their name and accession number (FW- freshwater, MAR- marine and SO- soil). Scale
690 bar = 0.02 substitution per site.

691

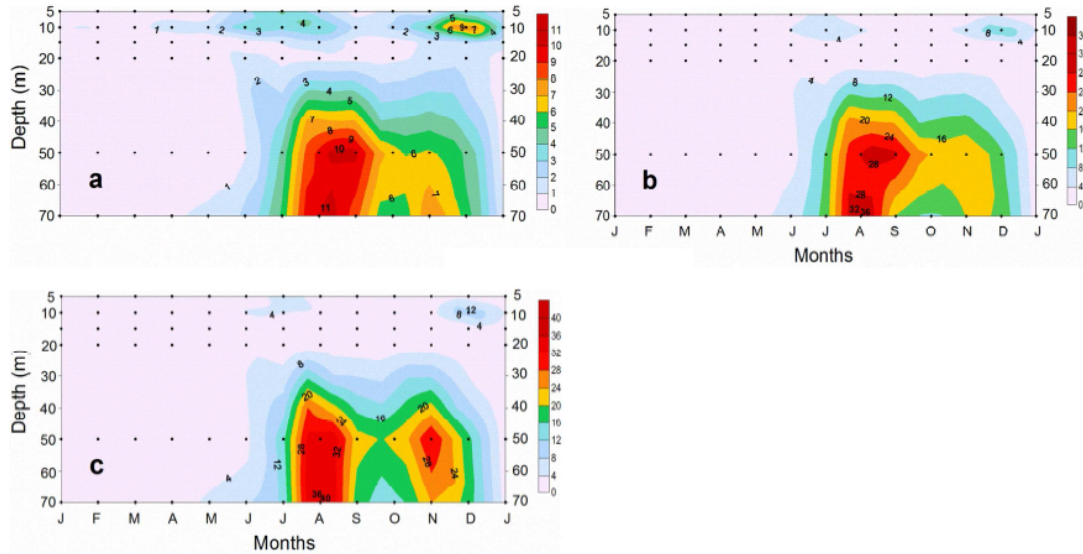


Figure 5: (a) Abundance of kinetoplastid cells targeted by KIN516 probe (10^2 cells ml^{-1}), (b) percentage of kinetoplastids to total eukaryotes, (c) percentage of kinetoplastids to total flagellates (HNF+ANF). Black dots represent sampling depth.